

05/05/99
JCS71 U.S. PTO
Frederick P. Fish
1855-1930
W.K. Richardson
1859-1951

FISH & RICHARDSON P.C.

A
Box Seg.
225 Franklin Street
Boston, Massachusetts
02110-2804
Telephone
617 542-5078
Facsimile
617 542-8906
Web Site
www.fr.com
JCS71 U.S. PTO
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Attorney Docket No.: 06501/030001

Box Patent Application

Assistant Commissioner for Patents
Washington, DC 20231

Presented for filing is a new original patent application of:

Applicant: HIROAKI YAMAMOTO
Title: METHOD FOR PRODUCING OPTICALLY ACTIVE
4-HALO-3-HYDROXYBUTYRIC ACID ESTER

Enclosed are the following papers, including those required to receive a filing date under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	52
Claims	4
Abstract	1
Signed Declaration [To Be Filed At A Later Date]	
Drawing(s)	4

Enclosures:

- Nucleotide and/or amino acid sequence listing including:
Computer readable copy.
Paper copy, 10 pages.
Verification statement, 1 page.
- A certified copy of the priority application will be filed at a later date.
- Postcard.

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FISH & RICHARDSON P.C.

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Under 35 USC §119, this application claims the benefit of foreign priority applications filed in Japan: serial number 10/126507, filed May 8, 1998; serial number 10/300178, filed October 21, 1998; and serial number 11/98205, filed April 5, 1999.

Basic filing fee	760.00
Total claims in excess of 20 times \$18.00	36.00
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Fee for multiple dependent claims	0.00
Total filing fee:	\$ 796.00

A check for the filing fee is enclosed. Please apply any other required fees or any credits to Deposit Account No. 06-1050, referencing the attorney docket number shown above.

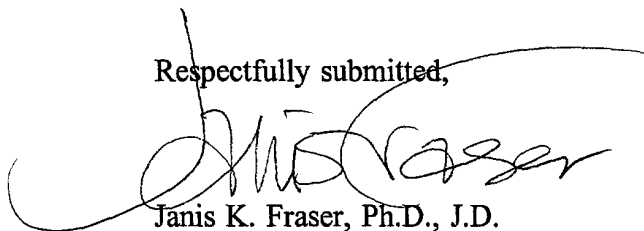
If this application is found to be incomplete, or if a telephone conference would otherwise be helpful, please call the undersigned at 617/542-5070.

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Please send all correspondence to:

Janis K. Fraser, Ph.D., J.D.
Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804

Respectfully submitted,



Janis K. Fraser, Ph.D., J.D.
Reg. No. 34,819

Enclosures

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APPLICATION
FOR
UNITED STATES LETTERS PATENT

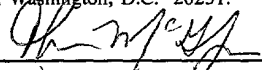
TITLE: **METHOD FOR PRODUCING OPTICALLY ACTIVE**
 4-HALO-3-HYDROXYBUTYRIC ACID ESTER

APPLICANT: **HIROAKI YAMAMOTO**

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Thomas McGlynn

METHOD FOR PRODUCING OPTICALLY ACTIVE
4-HALO-3-HYDROXYBUTYRIC ACID ESTER

FIELD OF THE INVENTION

The present invention relates to a method for preparing (S)-4-halo-3-hydroxybutyric acid ester using β -ketoacyl-acylcarrier-protein reductase comprising Type II fatty acid synthase or acetoacetyl-CoA reductase comprising poly- β -hydroxy fatty acid synthase.

BACKGROUND OF THE INVENTION

Known methods for preparing the optically active (S)-4-halo-3-hydroxybutyric acid ester include an asymmetric reduction method using 3- α -hydroxysteroid dehydrogenase (Japanese Patent Laid-Open Publication No. Hei 1-277494) and microorganisms such as baker's yeast (J. Am. Chem. Soc. 105, 5925-5926 (1983); Japanese Patent Laid-Open Publication No. Sho 61-146191). D-enzyme-1 and D-enzyme-2 have been reported as the enzyme of baker's yeast reducing 4-haloacetoacetic acid ester to produce (S)-4-halo-3-hydroxybutyric acid ester (J. Org. Chem. 56, 4778-4483 (1991)). Of these enzymes, D-enzyme-2 has been indicated to be the fatty acid synthase based on its molecular weight, etc. (J. Am. Chem. Soc. 107, 2993 (1985)).

However, in the synthesis of optically active (S)-4-halo-3-hydroxybutyric acid ester by reducing 4-haloacetoacetic acid ester using baker's yeast, the enzymatic activity is too low to produce the desired product in a high concentration. Furthermore, since baker's yeast has an enzyme reducing 4-haloacetoacetic acid ester to produce (R)-4-halo-3-hydroxybutyric acid ester, it is difficult to stably synthesize (S)-4-halo-3-hydroxybutyric acid ester with a high optical purity.

In addition, the fatty acid synthase mainly involved in the synthesis of (S)-4-halo-3-hydroxybutyric acid ester in baker's yeast has been reported to be quickly inhibited by SH reagents such as iodoacetamide, mercury, and p-(chloromercury)benzoic acid. Thus, the enzyme is expected to be inhibited by the substrate 4-haloacetoacetic acid ester and the product 4-halo-3-hydroxybutyric acid ester. Therefore, baker's yeast is not preferable for producing (S)-4-halo-3-hydroxybutyric acid ester in a large quantity.

It is also conceivable to synthesize (S)-4-halo-3-hydroxybutyric acid ester with a high optical purity by highly expressing the fatty acid synthase of baker's yeast with a high specific activity in heterologous microorganisms using genetic engineering techniques. However, the fatty acid synthase of baker's yeast is an extremely complex multicatalytic enzyme, in which α -subunit with the molecular weight of 208,000 consisting of 1,894 amino acid residues and β -subunit with the molecular weight of 229,000 consisting of 2,051 amino acid residues (J. Biol. Chem. 263, 12315-12325 (1988)) form an $\alpha_6\beta_6$ complex (J. Biol. Chem. 253, 4464-4475 (1978)), having eight different activities, besides the β -keto group reducing activity (β -ketoacyl-ACP reducing activity), including the acyl carrier protein (ACP) activity, ACP-S-acetyltransferase activity, ACP-S-malonyl transferase activity, β -ketoacyl-ACP synthase activity, β -hydroxyacyl-ACP dehydrogenase activity, enoyl-ACP reductase activity, and palmitoyl transferase activity. Therefore, it is not easy to highly express this synthase in heterologous microorganisms. For example, an attempt to express FAS1 and FAS2 in minicells of *E. coli* reportedly resulted in failure to detect a full length of the enzymes (Ann. Rev. Biochem. 52, 537-579 (1983)).

The domain for the β -ketoacyl-ACP reducing activity which is expected to exhibit the 4-haloacetoacetic acid ester reductase activity has been indicated to be located in the α -subunit of the fatty acid synthase based on the amino acid sequence. It has been reported, however, that, when the α -subunit was completely dissociated by freeze-thawing in a high salt concentration (Biochem. J. 109, 312-314 (1968)) and by lysine modification with dimethyl maleic anhydride, the subunit alone did not express the β -ketoacyl-ACP reducing activity (Eur. J. Biochem. 94, 189-197 (1979)). It has also been reported that ethyl acetoacetate reducing activity was not expressed by the fatty acid synthase with an $\alpha_6\beta_6$ structure but expressed only by that with an $\alpha_2\beta_2$ structure (Mw 800,000) (Eur. J. Biochem. 172, 633-639 (1988)). Therefore, it has not been clarified which domain of the fatty acid synthase is essential for the 4-haloacetoacetic acid reducing activity and how to efficiently prepare the structure ($\alpha_2\beta_2$) expressing the 4-haloacetoacetic acid ester reducing activity.

SUMMARY OF THE INVENTION

An objective of the present invention is to provide a method for efficiently producing (S)-4-halo-3-hydroxybutyric acid ester utilizing an enzyme constituting fatty acid synthase or that constituting the poly- β -hydroxy fatty acid biosynthetic system.

Fatty acid synthase is structurally classified into four types, IA, IB, IC, and II (Shin Seikagaku Jikken Koza (New Biochemical Experiment) 4, Shisitu (Lipids) I, p34-37). Animals including humans (Proc. Natl. Acad. Sci. USA 92, 8695 (1995)) have Type IA synthase comprising a homodimer (Mw about 500,000) of α -subunit (Mw about 250,000) having all of the above-described eight different activities

of fatty acid synthase. Yeasts including baker's yeast and fungi have Type IB synthase of an $\alpha_6\beta_6$ structure (Mw about 2,400,000) consisting of α -subunit (Mw about 210,000) and β -subunit (Mw about 200,000) expressing all the fatty acid synthase activities. Bacteria such as *Brevibacterium ammoniagenes* (Eur. J. Biochem. 247, 268 (1997)) and *Micobacterium smegmatis* (Physiol. Rev. 56, 339 (1976)) have Type IC synthase of an α_6 structure consisting of α -subunit (Mw about 250,000) having all the fatty acid synthase activities. Plants such as *Brassica napus* (Biochim. Biophys. Acta, 1120, 151 (1992)) and algae, bacteria such as *Escherichia coli*, *Actinomycetes*, and viruses have Type II synthase in which individual reactions of the fatty acid synthase are carried out with separate enzyme proteins.

Taking notice of β -ketoacyl-ACP reductase classified into Type II fatty acid synthase, among these various types of enzymes, which is simpler in the structure and functions, smaller in molecular weight (Mw of subunit about 20,000 to 40,000) as compared with Type I enzymes (IA, IB, and IC), and not inhibited by SH reagents, the present inventors have thought that, if the enzyme has the activity to reduce 4-haloaetoetic acid ester to synthesize (S)-4-halo-3-hydroxybutyric acid ester like Type IB fatty acid synthase of baker's yeast, it would be possible to produce (S)-4-halo-3-hydroxybutyric acid ester in a large quantity and create a microbial strain capable of producing (S)-4-halo-3-hydroxybutyric acid ester in high yield utilizing genetic engineering techniques.

Therefore, the present inventors have attempted the isolation of β -ketoacyl-ACP reductase constituting Type II fatty acid synthase to investigate its reducing activity toward 4-haloaetoetic acid ester.

Specifically, β -ketoacyl-ACP reductase genes of *Escherichia coli* or *Bacillus subtilis*, whose nucleotide sequences were known, were cloned by the polymerase chain reaction with chromosomal DNAs from respective bacteria as the template. The isolated gene was then introduced into *Escherichia coli* to highly express the enzyme therein to examine its 4-chloroacetoacetic acid ester reducing activity. As a result, the present inventors have found that the enzyme has an extremely high reducing activity and stereoselectivity toward 4-chloroacetoacetic acid ester.

In addition, while it has been reported that β -ketoacyl-ACP reductase from *Escherichia coli* showed an oxidizing activity specific to the D-stereoisomer of β -hydroxybutyl-ACP (J. Biol. Chem. 240, 618-621 (1965)), the present inventors have found that the enzyme as well as the enzyme derived from *Bacillus subtilis* showed almost no oxidizing activity to 4-chloro-3-hydroxybutyric acid ester of either configuration, but only reducing ethyl 4-chloroacetoacetate. Such a property is extremely advantageous for synthesizing an optically active (S)-4-halo-3-hydroxybutyric acid ester by asymmetric reduction because the reaction equilibrium is not rate-limiting.

Furthermore, the present inventors have found that genes encoding acetoacetyl-CoA reductase (generally designated phbB or phaB), one of the enzymes constituting the poly- β -hydroxy fatty acid (PHA) biosynthesis system, have homology to that encoding Type II fatty acid synthase (generally designated fabG), and examined whether these acetoacetyl-CoA reductases are capable of asymmetrically reducing 4-chloroacetoacetic acid ester to (S)-4-halo-3-hydroxybutyric acid ester like β -ketoacyl-ACP reductase. Specifically, the inventors

isolated the acetoacetyl-CoA reductase gene from *Ralstonia eutropha*, introduced the resulting gene to *Escherihia coli* to express it, and used the expressed acetoacetyl-CoA reductase to reduce 4-chloroacetoacetic acid ester. As a result, it has been found that the enzyme has a high reducing activity and stereoselectivity for producing (S)-4-chloro-3-hydroxybutyric acid ester.

The inventors also have found that acetoacetyl-CoA reductase, like β -ketoacyl-ACP reductase, shows almost no reactivity to either optical isomer of 4-chloro-3-hydroxybutyric acid ester, and practically functions as only the reductase, which is favorable to the synthesis of (S)-4-chloro-3-hydroxybutyric acid ester.

Furthermore, the present inventors have attempted to efficiently accelerate the reducing reaction cycle by regenerating the coenzyme (NADPH or NADH), which is consumed associated with the asymmetric reduction of 4-chloroacetoacetic acid ester in this reducing reaction system. First, the inventors introduced into *Escherihia coli* a gene encoding glucose dehydrogenase capable of regenerating the coenzyme (NADPH or NADH) and a gene encoding β -ketoacyl-ACP reductase, thereby obtaining E. coli strain capable of producing these enzymes. Then, ethyl 4-haloacetoacetate was reduced utilizing the thus-obtained transformant to measure the yield and the optical purity of ethyl (S)-chloro-3-hydroxybutyrate thus produced. As a result, it was found that the recombinant enzyme produced by the *Escherihia coli* strain has a high enzymatic activity quite suitable for producing ethyl (S)-chloro-3-hydroxybutyrate with a high optical purity.

The present invention relates to a method for producing (S)-4-halo-3-hydroxybutyric acid ester by reacting 4-halo-acetoacetic acid

ester or its derivatives with β -ketoacyl-ACP reductase constituting Type II fatty acid synthase or acetoacetyl-CoA reductase, one of the enzymes constituting the poly- β -hydroxy fatty acid biosynthesis system. More specifically, it relates to:

- (1) a method for producing (S)-4-halo-3-hydroxybutyric acid ester comprising asymmetrically reducing 4-halo-acetoacetic acid ester or its derivatives with β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase,
- (2) the method of (1), wherein said β -ketoacyl carrier protein reductase is derived from *Escherihia coli*,
- (3) the method of (1), wherein said β -ketoacyl carrier protein reductase is selected from the group consisting of:
 - (a) a protein comprising the amino acid sequence of SEQ ID NO: 1;
 - (b) a protein comprising a modified amino acid sequence of SEQ ID NO: 1 in which one or more amino acid residues are added, deleted, or substituted and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester; and
 - (c) a protein encoded by DNA hybridizable with the DNA comprising the nucleotide sequence of SEQ ID NO: 2 and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester,
- (4) the method of (1), wherein said β -ketoacyl-acyl carrier protein reductase is derived from microorganisms belonging to the genus *Bacillus*,
- (5) the method of (4), wherein said β -ketoacyl-acyl carrier protein reductase is derived from *Bacillus subtilis*,

(6) the method of (1), wherein said β -ketoacyl-acyl carrier protein reductase is selected from the group consisting of:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 5;

(b) a protein comprising a modified amino acid sequence of SEQ ID NO: 5 in which one or more amino acid residues are added, deleted, or substituted and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester; and

(c) a protein encoded by DNA hybridizable with the DNA comprising the nucleotide sequence of SEQ ID NO: 6 and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester,

(7) a method for producing (S)-4-halo-3-hydroxybutyric acid ester comprising asymmetrically reducing 4-halo-acetoacetic acid ester or its derivatives with acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid biosynthesis system,

(8) a method of (7), wherein said acetoacetyl-CoA reductase is derived from microorganisms belonging to the genus *Ralstonia*,

(9) a method of (8), wherein said acetoacetyl-CoA reductase is derived from *Ralstonia eutropha*,

(10) the method of (7), wherein said acetoacetyl-CoA reductase is selected from the group consisting of:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 9;

(b) a protein comprising a modified amino acid sequence of SEQ ID NO: 9 in which one or more amino acid residues are added, deleted, or substituted and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric

acid ester; and

(c) a protein encoded by DNA hybridizable with the DNA comprising the nucleotide sequence of SEQ ID NO: 10 and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester,

(11) the method of (1) or (7), wherein said 4-haloacetoacetic acid ester is 4-chloroacetoacetic acid ester,

(12) the method of (1) or (7), wherein said 4-haloacetoacetic acid ester is ethyl 4-chloroacetoacetate,

(13) the method of (1), wherein said method uses a microorganism capable of producing β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase and an enzyme catalyzing production of NAD(P)H from NAD(P)⁺,

(14) the method of (13), wherein said microorganism is a recombinant microorganism into which heterologous or homologous DNA encoding the β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase and heterologous or homologous DNA encoding an enzyme catalyzing production of NAD(P)H from NAD(P)⁺, and is capable of expressing both enzymes,

(15) the method of (14), wherein said microorganism is *Escherichia coli*,

(16) the method of any one of (13) through (15), wherein said enzyme catalyzing production of NAD(P)H from NAD(P)⁺ is glucose dehydrogenase,

(17) the method of (7), wherein said method uses a microorganism capable of producing acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid biosynthesis system and an enzyme catalyzing production of NAD(P)H from NAD(P)⁺,

(18) the method of (17), wherein said microorganism is a recombinant

microorganism into which heterologous or homologous DNA encoding acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid biosynthesis system and heterologous or homologous DNA encoding an enzyme catalyzing production of NAD(P)H from NAD(P)⁺ and capable of expressing both enzymes,

(19) the method of (18), wherein said microorganism is *Escherichia coli*, and

(20) the method of any one of (17) through (19), wherein said enzyme catalyzing production of NAD(P)H from NAD(P)⁺ is glucose dehydrogenase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the restriction map of plasmid pSE420D. P(trc) represents the trc promoter, T(rrnB) the rrnB T1T2 terminator, Amp the β -lactamase gene for ampicillin resistance, and ori the replication origin of plasmid.

Figure 2 shows the restriction map of plasmid pSG-ECR1. P(trc) represents the trc promoter, EcfabG the β -ketoacyl ACP reductase gene derived from *Escherichia coli*, BSGlcDH the glucose dehydrogenase gene derived from *Bacillus subtilis*, T(rrnB) the rrnB T1T2 terminator, Amp the β -lactamase gene for ampicillin resistance, and ori the replication origin of plasmid.

Figure 3 shows the restriction map of plasmid pSG-BSR1. P(trc) represents the trc promoter, BcfabG the β -ketoacyl ACP reductase gene derived from *Bacillus subtilis*, BSGlcDH the glucose dehydrogenase gene derived from *Bacillus subtilis*, T(rrnB) the rrnB T1T2 terminator, Amp the β -lactamase gene for ampicillin resistance, and ori the replication origin of plasmid.

Figure 4 shows the restriction map of plasmid pSG-AER1. P(trc)

represents the trc promoter, AephbB the acetoacetyl-CoA reductase gene derived from *Ralstonia eutropha*, BSGlcDH the glucose dehydrogenase gene derived from *Bacillus subtilis*, T(rrnB) the rrnB T1T2 terminator, Amp the β -lactamase gene for ampicillin resistance, and ori the replication origin of plasmid.

DETAILED DESCRIPTION OF THE INVENTION

The method for producing (S)-4-halo-3-hydroxybutyric acid ester of the present invention uses the enzyme constituting Type II fatty acid synthase (EC1.1.1.100) or acetoacetyl-CoA reductase (EC 1.1.1.36) constituting the PHA biosynthesis system. The enzyme (EC 1.1.1.100) constituting Type II fatty acid synthase is simpler in the structure and functions, smaller in the molecular weight (Mw of subunit about 20,000 to 40,000), and not inhibited by SH reagents as compared with Type IA synthase comprising a homodimer of α -subunit, Type IB synthase of an $\alpha_6\beta_6$ structure consisting of α -subunit and β -subunit, and Type IC synthase of an α_6 structure of α -subunit, which have all of various activities of fatty acid synthase. Therefore, Type II synthase is advantageous and preferable for large quantity production of (S)-4-halo-3-hydroxybutyric acid ester and construction of a microbial strain capable of high level production of (S)-4-halo-3-hydroxybutyric acid ester.

The source of the synthase used is not particularly limited. In addition to β -ketoacyl-ACP reductase derived from *Escherichia coli* (SEQ ID NO: 1, J. Biol. Chem. 267, 5751-5754 (1992)) and that derived from *Bacillus subtilis* (SEQ ID NO: 5, J. Bacteriol. 178, 4794-4800 (1996)), and enzymes derived from other various organisms can be used. The enzymes derived from other organisms include β -ketoacyl-ACP reductase

derived from *Actinobacillus actinomycetemcomitans* (Biochem. Biophys. Res. Commun, 230, 220-225 (1997)), *Bacillus subtilis* (J. Bacteriol. 178, 4794-4800 (1996)), *Escherichia coli* (J. Biol. Chem. 267, 5751-5754 (1992)), *Mycobacterium bovis* (Science 267, 227-230 (1994)), *Mycobacterium smegmatis* (DDBJ Accession number U66800), *Mycobacterium tuberculosis* (Mol. Microbiol. 15, 1009-1015 (1995)), *Propionibacterium shermanii* (J. Gen. Microbiol. 127, 121-129 (1981)), *Streptococcus pneumoniae* (W097/43303), *Synechocystis* sp. (DNA Res. 3, 109-136 (1996)), *Thermotoga maritima* (J. Bacteriol. 178, 248-257 (1996)), *Vibrio harveyi* (J. Bacteriol. 178, 571-573 (1996)), *Haemophilus influenza* (Science 269, 469-512 (1995)), etc. β -ketoacyl-ACP reductases derived from plants include those derived from *Allium porrum* (Plant Physiol. 115, 501-510 (1997)), *Arabidopsis thaliana* (Biochem. J. 283, 321-326 (1992), Plant Physiol. 115, 501-510 (1997)), *Brassica napus* (W096/02652)), *Carthamus tinctorius* (Arch. Biochem. Biophys. 217, 144-154 (1982)), *Cuphea lanceolata* (Mol. Gen. Genet. 233, 122-128 (1992)), *Hordeum vulgare* (Plant Physiol. 115, 501-510 (1997)), *Persea americana* (Biochem. J. 271, 713-720 (1990)), carrot (Arch. Biochem. Biophys. 300, 157-163 (1993)), *Euglena gracilis* (J. Biol. Chem. 255, 1504-1508 (1980)), *Spinacia oleracea* (Plant Physiol. 69, 1257-1262 (1982)), *Zea mays* L. (Plant Physiol. 115, 501-510 (1997)), etc.

A gene encoding β -ketoacyl-ACP reductase can be isolated utilizing, for example, hybridization techniques. A β -ketoacyl-ACP reductase gene derived from various organisms can be isolated by hybridization, under the stringent conditions, with the DNA encoding β -ketoacyl-ACP reductase derived from *Escherichia coli* (SEQ ID NO: 2) and that from *Bacillus subtilis* (SEQ ID NO: 6), or DNAs prepared from

other organisms using the portions thereof as the probe. The polymerase chain reaction can also be utilized to isolate the desired gene. For example, primers are designed based on the highly homologous region in the gene encoding β -ketoacyl-ACP reductase (e.g. NADPH-binding region, 10th to 34th amino acid residues of β -ketoacyl-ACP reductase from *Escherichia coli*) and, using the resulting primers and the chromosomal DNA or cDNA of a target organism as the template, the polymerase chain reaction is performed to isolate the gene encoding β -ketoacyl-ACP reductase from various organisms.

Poly- β -hydroxy fatty acid (PHA) is known to accumulate in more than 100 varieties of prokaryotic microorganisms including the genus *Alcaligenes*, *Aphanothece*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhodospirillum*, and *Actinomyces*. The PHA biosynthetic system comprises 3-ketothiolase, acetoacetyl CoA reductase and PHA synthase. Among them, acetoacetyl CoA reductase comprises a tetramer of the subunit with Mw 20,000 to 40,000 and preferably uses reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the coenzyme for the reducing reaction. It can also employ a more economical and highly stable reduced nicotinamide adenine dinucleotide (NADH) as the coenzyme and thus is industrially advantageous.

Any acetoacetyl CoA reductases can be used in the present invention regardless of their origin so far as they are acetoacetyl CoA reductase participating in the PHA biosynthesis. Examples thereof include acetoacetyl CoA reductase derived from *Acinetobacter* sp. RA3849 (J. Bacteriol. 177, 4501-4507 (1995)), *Ralstonia eutropha* (previously called *Alcaligenes eutrophus*, FEMS Microbiol. Lett. 52, 259-264 (1988)), *Alcaligenes latus* (J. Microbiol. Biotechnol. 6, 425-431 (1996)),

Alcaligenes sp. SH-69 (DDBJ Accession No. AF002014), *Azospirillum brasilense* (J. Gen. Microbiol. 136, 1191-1196 (1990), Mol. Gen. Genet. 231, 375-384 (1992)), *Azotobacter beijerinckii* (Biochem. J., 134, 225-238 (1973)), *Bacillus megaterium* (Can. J. Microbiol. 41 (Suppl. 1), 77-79 (1995)), *Chromatium vinosum* D strain (Eur. J. Biochem. 209, 135-150 (1992)), *Ectothiorhodospira shaposhnikovii* (Appl. Microbiol. Biotechnol. 40, 292-300 (1993)), *Lupinus luteus* (Plant Soil, 56, 379-390 (1980)), *Methylobacterium extorquens* (FEMS Microbiol. Lett. 156, 275-279 (1997)), *Methylobacterium rhodesianum* MB 126 (Arch. Microbiol. 161, 277-280 (1994)), *Paracoccus denitrificans* (FEMS Microbiol. Rev. 103, 257-264 (1992), FEMS Microbiol. Lett. 133, 85-90 (1995)), *Pseudomonas* sp. (DDBJ Accession No. Z80156), *Rhizobium lupini* (Fiziol. Rast. (Moscow) 27, 544-550 (1980)), *Rhizobium meliloti* 41 or *Sinorhizobium meliloti* (Microbiology, 141, 2553-2559 (1995)), *Rhodococcus ruber* NCIMB 40126 (FEMS Microbiol. Rev., 103, 93-101 (1992)), *Synechococcus* sp. (Japanese Patent Laid-open Publication No. Hei 8-187085), *Syntrophomonas wolfei* subsp. *wolfei* (Arch. Microbiol. 159, 16-20 (1993)), *Thiocapsa pfennigii* (Appl. Microbiol. Biotechnol. 40, 292-300 (1993)), and *Zoogloea ramigera* I-16-M (Arch. Microbiol. 114, 211-217 (1977), J. Biol. Chem. 262, 97-102 (1987)).

If the nucleotide sequence of the gene encoding acetoacetyl-CoA reductase is known, the coding region can be isolated using the polymerase chain reaction (PCR). The acetoacetyl-CoA reductase gene can also be isolated from various organisms by hybridizing, under the stringent conditions, the target DNA with DNA encoding acetoacetyl-CoA reductase derived from, for example, *Ralstonia eutropha* (SEQ ID NO: 10, the amino acid sequence of the enzyme is shown in SEQ ID NO: 9) or DNA

prepared from other organisms using the portions of the above enzyme-encoding DNA as the probe. Furthermore, it is also possible to isolate the acetoacetyl-CoA reductase gene from various organisms utilizing the polymerase chain reaction (PCR) with primers designed based on a highly homologous region (such as the NADPH-binding region) of the gene encoding acetoacetyl-CoA reductase and the chromosomal DNA or cDNA from a target organism as the template.

In the method of the present invention, may be used not only naturally-occurring enzymes but also variant enzymes having modified amino acid sequence of the natural enzyme so far as the variant enzymes are functionally equivalent to the natural enzyme. Such modification of the amino acid sequence can be made by the method using BAL31 exonuclease III, Kunkel method, and PCR, which are well known to those skilled in the art (Labomanual Genetic Engineering, 3rd ed., p219-230, Maruzen). Since the amino acid substitution may occur spontaneously, not only enzymes having amino acid artificially modified but also enzymes with spontaneously modified amino acid sequence can be used in the present invention.

In addition, the enzyme genes used in the method of the present invention include the gene having homology to β -ketoacyl-ACP reductase or acetoacetyl-CoA reductase and encoding Type II synthase of polyketides biosynthesized through the system similar to that of fatty acid synthesis and PHB synthesis (e.g. the open reading frame (ORF)-3 gene of the actI gene derived from *Sachropolyspora hirsuta*, Mol. Gen. Genet. 240, 146-150 (1993)), ORF-5 in the gene having homology to the above actI gene derived from *Streptomyces cinnamonensis* and putatively participating in the biosynthesis of monensin (Mol. Gen. Genet. 234,

254-264 (1992)), actIII that is a gene for actinorhodin biosynthesis derived from *Streptomyces coelicolor* (Gene 74, 305-320 (1988)), aknA that is a gene for Aklavinone biosynthesis derived from *Streptomyces galilaeus* (J. Bacteriol., 176, 2473-2475 (1994)), dauB that is a gene for daunomyin biosynthesis derived from *Streptomyces* sp. C5 (J. Bacteriol., 176, 6270-6280 (1994)), ORF-5 of the gene for granaticin polyketide synthase putative ketoacyl reductase 1 derived from *Streptomyces violaceoruber* Tu22, ORF-6 of the gene for the granaticin polyketide synthase putative ketoacyl reductase 2 (EMBO J., 8, 2717-2725 (1989), etc.), nodG that is a gene involved in the nodulation derived from *Rhizobium meliloti* RCR2011 (Nucleic Acids Res. 14, 7453-7472 (1986)), and hetN that is a gene involved in the heterocyst formation derived from *Anabaena* sp. (PCC 7120) (J. Bacteriol. 176, 2282-2292 (1994)). These can be preferably used as long as enzymes (such as β -ketoacyl reductase), products of these genes, have the activity to reduce 4-halo-acetoacetic acid ester to produce (S)-4-halo-3-hydroxybutyric acid.

In the present invention, "asymmetric reduction of 4-halo-acetoacetic acid ester or its derivatives using β -ketoacyl-acyl carrier protein (ACP) reductase or acetoacetyl-CoA reductase" is not necessarily performed using the purified enzyme. Microorganisms and plants containing said enzyme, or their treated products can also be used. Especially, it is preferable to use organisms transformed with a heterologous or homologous gene encoding β -ketoacyl-ACP reductase or acetoacetyl-CoA reductase using genetic engineering techniques to enable high level expression of the enzyme or the treated products of the organisms. If the organism having β -ketoacyl-ACP reductase or

acetoacetyl-CoA reductase also possesses the enzyme reducing 4-halo-acetoacetic acid ester or its derivatives to synthesize (R)-4-halo-3-hydroxybutyric acid ester, such a organism is preferably mutated to delete these (R)-enatiomer generating enzymes by the natural or artificial mutation or recombinant DNA techniques.

The host microorganisms used the present invention are not particularly limited as long as they can be transformed with DNA encoding the polypeptide having the activity of β -ketoacyl-ACP reductase or acetoacetyl-CoA reductase and express these enzyme activities. Specific examples thereof include bacteria for which the host vector system has been developed, such as the genera *Escherichia*, *Bacillus*, *Pseudomonas*, *Serratia*, *Brevibacterium*, *Corynebacterium*, *Streptococcus*, and *Lactobacillus*, yeasts such as *Saccharomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Yarrowia*, *Trichosporon*, *Rhodospiridium*, *Hansenula*, *Pichia*, and *Candida*, and fungi such as the genera *Neurospora*, *Aspergillus*, *Cephalosporium*, and *Trichoderma*.

Transformants can be prepared by techniques conventionally used in the field of molecular biology, biotechnology, and genetic engineering (e.g., Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratories). In order to express the gene for β -ketoacyl-ACP reductase or acetoacetyl-CoA reductase of the present invention in microorganisms and the like host, it is necessary to first introduce the gene into a plasmid or phage vector that can be stably present in microorganisms to transcript and translate the genetic information. For that purpose, as the unit for controlling the transcription and translation, a promoter can be incorporated upstream of the 5'-side of

the gene and a terminator downstream of the 3'-side of the gene. Any promoter and terminator can be used so far as they function in microorganisms to be used as the host. Vectors, promoters, and terminators functioning in various microorganisms are described in detail in "Biseibutugaku Kisokoza (Fundamental Microbiology), 8, Idenshikogaku (Genetic Engineering), Kyoritsu", especially those usable in yeast in Adv. Biochem. Eng. 43, 78-102 (1980) and Yeast 8, 423-488 (1992).

For example, in the genus *Escherichia*, especially in *Escherichia coli*, pBR and pUC series can be used as the plasmid vector. Examples of the promoters include those derived from *lac* (β -galactosidase), *trp* (tryptophan operon), *tac* (*lac* and *trp* fused), phage λ PL, and PR. The terminators include *trpA* terminator and *rrnB* ribosomal terminator.

In the genus *Bacillus*, the plasmid pUB110 pC194 series can be used as the vector. These vectors can be integrated into chromosomes. as the promoter and the terminator, those for *apr* (alkaline protease), *npr* (neutral protease), and *amy* (.-amylase) can be used.

In the genus *Pseudomonas*, the host-vector system has been developed in *Pseudomonas putida*, *Pseudomonas cepacia*, etc. Vectors with a broad host spectrum such as pKT240 (comprising the gene required for the autonomous replication derived from RSF1010 or the like) developed based on the plasmid TOL participating in the degradation of toluene compounds can be used. An example of the promoter and terminator that can be used is those for the lipase gene (Japanese Patent Laid-Open Publication No. Hei 5-284973).

In the genus *Brevibacterium*, especially in *Brevibacterium lactofermentum*, the plasmid vector such as pAJ43 (Gene 39, 281 (1985))

can be used. In this vector, the promoters and terminators employed in *Escherichia coli* can be used.

In the genus *Corynebacterium*, especially in *Corynebacterium glutamicum*, the plasmid vectors such as pCS11 (Japanese Patent Laid-Open Publication No. Sho 57-183799) and pCB101 (Mol. Gen. Genet. 196, 175 (1984)) can be used.

In the genus *Streptococcus*, pHV1301 (FEMS Microbiol. Lett. 26, 239 (1985)) and pGK1 (Appl. Environ. Microbiol. 50, 94 (1985)) can be used as the plasmid vector.

In the genus *Lactobacillus*, vector pAM β 1 (J. Bacteriol. 137, 614 (1979)) developed for the genus *Streptococcus* and the promoter used in *Escherichia coli* can be employed.

In the genus *Saccharomyces*, especially in *Saccharomyces cerevisiae*, the plasmid YRp series, YEp series, YCp series, and YIp series can be used. The integration vector that utilizes the homologous recombination with the ribosomal RNA present in multicopies in chromosome (EP 537456) is extremely useful because it allows to integrate muticopies of a gene and stably maintain them. The promoters and terminators that can be used in this yeast are those for ADH (alcohol dehydrogenase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PHO (acidic phosphatase), GAL (β -galactosidase), PGK (phosphoglycerate kinase), and ENO (enolase).

In the genus *Kluyveromyces*, especially in *Kluyveromyces lactis*, examples of the vectots include the plasmid 2 μ m series derived from *Saccharomyces cerevisiae*, pKD1 series (J. Bacteriol. 145, 382-390 (1981)), plasmids derived from pKGl1 concerned with the killer activity, KARS series which is the autonomous replication gene in the genus

Kluyveromyces, and the vector plasmid capable of integrating to chromosome by the homologous recombination with ribosomal RNA (EP 537456). Promoters and terminators derived from ADH, PGK, etc. can be used.

In the genus *Schizosaccharomyces*, the vectors to be used are ARS (the autonomous replication-related gene) derived from *Schizosaccharomyces pombe* and the plasmid vector containing the selection marker complementing the autotrophy derived from *Saccharomyces cerevisiae* (Mol. Cell Biol. 6, 80 (1986)). ADH promoter derived from *Schizosaccharomyces pombe* can be used (EMBO J. 6, 729 (1987)).

In the genus *Zygosaccharomyces*, the plasmid vector based on pSB3 (Nucleic Acids Res. 13, 4267 (1985)) derived from *Zygosaccharomyces rouxii* can be used. Examples of the promoters are PHO5 promoter derived from *Saccharomyces cerevisiae*, GAP-Zr (glyceraldehyde-3-phosphate dehydrogenase) promoter derived from *Zygosaccharomyces rouxii* (Agri. Biol. Chem. 54, 2521 (1990)).

In the genus *Hansenula*, the host vector has been developed in *Hansenula polymorpha*. The autonomous replication-related genes HARS1 and HARS2 derived from *Hansenula polymorpha* can be used as the vector. Since they are relatively unstable, the multicopy integration to chromosome is effective (Yeast 7, 431-443 (1991)). AOX promoter (alcohol oxidase) induced by methanol and FDH (formate dehydrogenase) promoter can be used.

In the genus *Pichia*, the host-vector system has been developed in *Pichia pastoris* utilizing the genes (PARS1 and PARS2) involved in the autonomous replication for *Pichia* (Mol. Cell. Biol. 5, 3376 (1985)).

In this vector, the potent promoter such as AOX inducible by the high concentration culture and methanol can be used (Nucleic Acids Res. 15, 3859 (1987)).

In the genus *Candida*, the host-vector system has been developed in *Candida maltosa*, *Candida albicans*, *Candida tropicalis*, *Candida utilis*, *Candida boidinii*, etc (Japanese Patent Laid-Open Publication No. Hei 5-344895) . *Candida maltosa*-derived ARS has been cloned (Agri. Biol. Chem. 51, 1587 (1987)) and used to develop a vector. In *Candida utilis*, a potent promoter has been developed for the vector capable of chromosomal integration (Japanese Patent Laid-Open Publication No. Hei 8-173170).

In the genus *Aspergillus*, *Aspergillus niger*, *Aspergillus oryzae*, etc. have been most comprehensively studied among fungi. Plasmids derived from these species and vectors capable of chromosomal integration are available. Promoters for the extracellular proteases and amylases can be used (Trends in Biotechnology 7, 283-287 (1989)).

In the genus *Trichoderma*, the *Trichoderma reesei*-based host-vector system has been developed. In this vector the promoter for the extracellular cellulase gene can be used (Biotechnology 7, 596-603 (1989)).

The culturing of transformants and purification of recombinant proteins from transformants can be performed by the conventional method known to those skilled in the art.

The substrate for the enzyme in the present invention is 4-halo-acetoacetic acid ester or its derivatives such as those with a substituent at the α -position. Preferable substrates are 4-chloroacetoacetic acid ester and ethyl 4-chloroacetoacetate. The

substrate concentration used in the method of this invention ranges usually from 0.1 to 50%, preferably from 1 to 20%. The enzyme amount used ranges usually from 0.01 to 500 U/ml of the reaction mixture, preferably from 0.1 to 50 U/ml. The coenzyme (NADPH or NADH) required by the enzyme is added in the reaction system if necessary in an amount of 0.00001 to 5 equivalents, preferably 0.0001 to 1 equivalent, with respect to the amount of the substrate. A buffer solution (for example, a phosphate buffer) can be used as a solvent in the reaction system to maintain the pH. A aqueous two-phase reaction system containing 10 to 90% organic solvent such as octane, hexane, toluene, ethyl acetate, n-butyl acetate, and chloroform can also be used. The reaction temperature is usually 4 to 50°C, preferably 10 to 30°C. pH is adjusted to usually 3 to 9, preferably 4 to 8. The reaction product, (S)-4-halo-3-hydroxybutyric acid ester, can be extracted by an organic solvent capable of dissolving the product well such as ethyl acetate and octane and purified by a method such as distillation.

In the above-described reaction, NADP^+ produced from NADPH during the reducing reaction is suitably converted to NADPH (acetoacetyl-CoA reductase and β -ketoacyl reductase involved in the biosynthesis of PHA and polyketides utilize not only NADPH but also NADH). The regeneration of the coenzymes can be performed utilizing the NAD(P)^+ reducing ability (such as the glycolytic pathway) of microorganisms. The NAD(P)^+ reducing ability can be increased by supplementing the reaction system with glucose or ethanol or by adding a microorganism capable of generating NAD(P)H from NAD(P)^+ , treated products thereof, or the enzyme with such an activity. For example, NAD(P)H can be regenerated using microorganisms having glucose dehydrogenase, malate dehydrogenase,

glutamate dehydrogenase, formate dehydrogenase, alcohol dehydrogenase, amino acid dehydrogenase, and/or glycerol dehydrogenase, treated products thereof, or the purified enzymes. Furthermore, the microorganism capable of producing β -ketoacyl reductase or acetoacetyl-CoA reductase can be genetically engineered to highly express these enzymes with the NAD(P)H regenerating activity, and the resulting transformant or the treated product thereof can be used in the method of the present invention.

In the present invention, it is also possible to use microorganisms capable of producing both acetoacetyl-CoA reductase and the NAD(P)H regenerating enzyme or both β -ketoacyl reductase and the NAD(P)H regenerating enzyme, or the treated products thereof. Such microorganisms can further genetically engineered to highly express these enzymes and the resulting microorganisms or their treated products can be used in the method of the present invention.

In the method of producing (S)-4-halo-3-hydroxybutyric acid ester using the microorganism capable of producing both acetoacetyl-CoA reductase and the NAD(P)H regenerating enzyme or both β -ketoacyl reductase and the NAD(P)H regenerating enzyme, or the treated products thereof, 1/50 to 1/20 volume, preferably 1/10 to 1/5 volume, based on the reaction mixture, of the culture, microbial cells recovered from the culture, or treated products thereof can be used. NAD⁺ or NADP⁺ can be added to the reaction mixture if necessary in an amount of 0.00001 to 5 equivalents, preferably 0.0001 to 1 equivalent, with respect to the amount of the substrate.

The reaction can also be performed using a bioreactor in which the microorganism producing the two enzymes or treated products thereof

is immobilized on κ -carrageenan, acrylamide, polyurethane, chitin, or the like carrier. The reaction using the bioreactor can be performed by continuously supplying 4-halo-acetoacetic acid ester as the substrate, the substrate for regenerating the coenzyme (e.g. glucose when glucose dehydrogenase is used as the enzyme for regenerating the coenzyme), and if required, a buffer for controlling pH, and NAD^+ or NADP^+ as the coenzyme, or repeatedly using the reaction mixture.

The present invention provides a method of producing the optically active (S)-4-halo-3-hydroxybutyric acid ester using β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase or acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid biosynthesizing system. β -Ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase is especially advantageous for constructing a highly productive system using genetic recombination techniques, enabling a more efficient production of the optically active (S)-4-halo-3-hydroxybutyric acid ester as compared with the prior art techniques.

The present invention will be described in more detail with reference to examples, but is not construed to be limited to these examples.

EXAMPLE 1

Isolation of β -ketoacyl-ACP Reductase Gene from *Escherichia coli*

Escherichia coli JM109 strain was cultured in the LB medium (containing Bacto-Tryptone 10 g, Bacto-Yeast extract 5 g, and NaCl 10 g/l) and the chromosomal DNA was prepared from the bacteria thus obtained using a Qiagen Genomic-tip (Qiagen). For the PCR cloning of the β -ketoacyl-ACP reductase gene (fabG) (J. Biol. Chem. 267, 5751-5754

(1992)) of *Escherichia coli*, the primers ECR-ATG1 (5'-AAAGGATCCAACAATGAATTTTGAAGGAAAAATCGC-3', SEQ ID NO: 3) and ECR-TAG1 (5'-TGCCTCGAGTTATCAGACCATGTACATCCCGC-3', SEQ ID NO: 4) were synthesized based on the nucleotide sequences at the 5'- and 3'-ends of the structural gene. Using the chromosomal DNA of *Escherichia coli* as the template, and the ECR-ATG1 and ECR-TAG1 primers, 30 cycles of PCR (95°C for 30 sec, 50°C for 1 min, and 75°C for 2 min) were performed to obtain the amplified DNA fragments.

The resulting DNA fragments were digested with restriction enzymes BamHI and XhoI. The plasmid vector pSE420 (Invitrogen) was digested with NcoI and BamHI, treated with the Klenow fragment, and subjected to the self-cyclization reaction to obtain the plasmid pSE420B. This pSE420B was digested with BamHI and XhoI, and ligated to the above PCR amplified fragments digested with the same two restriction enzymes using T4 DNA ligase to obtain the plasmid pSE-ECR1. The DNA insert in the plasmid thus obtained were sequenced and identified as the *fabG* gene. The nucleotide sequence of *fabG* is shown in SEQ ID NO: 2 and the amino acid sequence of the protein encoded by the gene in SEQ ID NO: 1.

EXAMPLE 2

Expression of β -ketoacyl-ACP Reductase Gene from *Escherichia coli*

Escherichia coli HB101 strain was transformed with pSE-ECR1 and the resulting transformant (*E. coli* HB101 (pSE-ECR1)) was cultured in the LB medium (5 ml) containing ampicillin (50 mg/ml) overnight. IPTG was added to the medium to 0.1 mM and the culture was further incubated for 5 h. Bacterial cells thus obtained were collected, disrupted with a Minibeatbeater 8 (BIOSPEC), and centrifuged to obtain the supernatant as the cell-free extract.

EXAMPLE 3

Reducing Activity of β -Ketoacyl-ACP Reductase from *Escherichia coli*

The reducing activity of the cell-free extract obtained in Example 2 was assayed using ethyl 4-chloroacetoacetate, ethyl acetoacetate, and acetoacetyl-CoA as the substrate.

A reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 0.2 mM NADPH, and 20 mM the substrate (0.2 mM when acetoacetyl-CoA was used as the substrate) and the enzyme was allowed to react at 25°C. One unit of the enzyme was defined as the amount of the enzyme catalyzing the decrease of 1 μ mol of NADPH for 1 min. The results are shown in Table 1. In this table, ECAA represents ethyl 4-chloroacetoacetate, EAA ethyl acetoacetate, and AASCoA acetoacetyl-CoA, R the reducing activity, and DH the dehydrogenase activity.

Table 1

	NADPH- ECAA-R	NADPH- AASCoA-R	NADPH- EAA-R	NADP ⁺ -R- ECHB-DH	NADP ⁺ -S- ECHB-DH
Cell- free extract	100%	116%	1.90%	0.010%	0.004%

The cell-free extract expressing β -ketoacyl-ACP reductase showed the NADPH-dependent ethyl 4-chloroacetoacetate-reducing activity, and its specific activity was 0.90 U/mg protein. In contrast, the HB101 strain containing no plasmid pSE-ECR1 showed almost no ethyl 4-chloroacetoacetate-reducing activity. The extract showed approximately the same activity for acetoacetyl-CoA as that for ethyl

4-chloroacetoacetate, while almost no activity for ethyl acetoacetate having no chlorine group.

EXAMPLE 4

Oxidizing Activity of β -Ketoacyl-ACP Reductase derived from *Escherichia coli*

The oxidizing activity of the cell-free extract obtained in Example 2 was assayed using ethyl (S)- or (R)-4-chloro-3-hydroxybutyrate as the substrate. The oxidizing reaction was performed by incubating a reaction mixture containing 50 mM Tris-HCl buffer (pH 9.0), 2.5 mM NADP⁺, 20 mM the substrate, and the enzyme at 25°C. One unit of enzyme was defined as the amount of enzyme catalyzing the increase of 1 μ mol of NADPH for min. The results are shown in Table 1. As shown in Table 1, the extract showed almost no oxidizing activity for either substrate. In this table, ECHB represents ethyl 4-chloro-3-hydroxybutyrate.

EXAMPLE 5

Stereoselectivity of β -Ketoacyl-ACP Reductase derived from *Escherichia coli* for Ethyl 4-chloro-3-hydroxybutyrate

A reaction mixture (1 ml) containing 200 mM potassium phosphate buffer (pH 6.5), 146 mM NADPH, 2% ethyl 4-chloroacetoacetate (122 mM), and β -ketoacyl-ACP reductase (2 U) prepared in Example 2 was incubated at 20°C overnight. Aliquot of the reaction solution was diluted 2-fold with 0.1 N HCl and ethyl 4-chloro-3-hydroxybutyrate contained in the dilution was determined by gas chromatography. Gas chromatography was performed with a Thermon 3000 chromosolve W (2 m, Shinwakako) under the conditions of the column temperature of 150°C and the detection temperature of 250°C using a flame ionization detector (FID). As a result, the concentration of ethyl 4-chloro-3-hydroxybutyrate was found

to be 13.3 g/l with a yield of 66.3%.

Optical purity was assayed by extracting ethyl 4-chloro-3-hydroxybutyrate from the reaction mixture with ethyl acetate, removing the solvent, and subjecting the residue to the high-performance liquid chromatography using an optical resolution column (column, chiracel OD (Daicel Chemical)); mobile phase, n-hexane/isopropanol (9/2); RI detection; flow rate, 0.5 ml/min). As a result, the optical purity was 96.5% ee (S) or more.

EXAMPLE 6

Synthesis of Ethyl 4-chloro-3-hydroxybutyrate by β -ketoacyl-ACP Reductase derived from *Escherichia coli*

A reaction solution (1 ml) containing 200 mM potassium phosphate buffer solution (pH 6.5), 2% ethyl 4-chloro-acetoacetate (122 mM), 1.0 mM NADPH, β -ketoacyl-ACP reductase (1 U), 250 mM glucose, and glucose dehydrogenase (Wako Pure Chemical) (10 U) was incubated at 25°C for 16 h. Analysis was performed in the same manner as in Example 5, indicating that ethyl 4-chloro-3-hydroxybutyrate with the optical purity of 95.4% ee (S) or more was synthesized in a yield of 98.4%.

EXAMPLE 7

Isolation of β -ketoacyl-ACP Reductase gene from *Bacillus subtilis*

Bacillus subtilis BGSC 1A1 strain was cultured in the LB medium (containing Bacto-Tryptone 10g, Bacto-Yeast extract 5 g, and NaCl 10 g/l), and the chromosomal DNA was prepared from the microbial cells thus obtained using a Qiagen Genomic-tip (Qiagen). For the PCR cloning of the β -ketoacyl-ACP reductase gene (fabG) of *Bacillus subtilis*, the primers BSR-ATG1 (5'-GGACCATGGATATGCTTAATGATAAAACGGCTA-3', SEQ ID NO:

7) and BSR-TAA1 (5'-GAGAAGCTTCTCGAGTTACATCACCATTCCGCCG-3', SEQ ID NO: 8) were synthesized based on the sequences at the 5'- and 3'-ends of the structural gene. Using the chromosomal DNA of *Bacillus subtilis* as the template and the BSR-ATG1 and BSR-TAA1 primers, 30 cycles of PCR (95°C for 30 sec, 50°C for 1 min, and 75°C for 2 min) were performed to obtain the amplified DNA fragments.

The DNA fragments thus obtained were digested with NcoI and HindIII. The plasmid vector pSE420 (Invitrogen) was digested with NcoI and HindIII, and linked to the above PCR-amplified fragments digested with the same two restriction enzymes using T4 DNA ligase to obtain the plasmid pSE-BSR1. The DNA inserts in the plasmid thus obtained were sequenced, identifying them to be the *fabG* gene except for one amino acid substitution. Namely, GCT corresponding to Ala at the position 25 according to the database (DDBJ (DNA Data Bank of Japan, Accession No. U59433) was replaced by GAT encoding Asp at the position 25. The nucleotide sequence of the *fabG* gene thus obtained is shown in SEQ ID NO: 6, the amino acid sequence of the protein encoded by the gene in SEQ ID NO: 5.

EXAMPLE 8

Expression of β -ketoacyl-ACP Reductase Gene derived from *Bacillus subtilis*

Escherichia coli HB101 strain was transformed with pSE-BSR1 and the resulting transformant (*E. coli* HB101 (pSE-BSR1)) was cultured in the LB medium (7 ml) containing ampicillin (50 mg/ml) overnight. IPTG was added to the medium to 0.1 mM and the culture was further incubated for 5 h. Bacterial cells thus obtained were collected, disrupted with a Minibeatbeater 8 (BIOSPEC), and centrifuged to obtain the supernatant

as the cell-free extract.

EXAMPLE 9

Reducing Activity of β -Ketoacyl-ACP Reductase
derived from *Bacillus subtilis*

The reducing activity of the cell-free extract obtained in Example 8 was assayed using ethyl 4-chloroacetoacetate and acetoacetyl-CoA as the substrate in the same manner as in Example 3.

The results are shown in Table 2. In this table, ECAA represents ethyl 4-chloroacetoacetate and AASCoA acetoacetyl-CoA.

Table 2

Substrate Coenzyme	ECAA		AASCoA	S-ECHB	R-ECHB
	NADPH	NADH	NADPH	NADP ⁺	NADP ⁺
U/mg	0.133	0	0.030	0.002	0.002
Relative activity	100%	0.0%	22.3%	1.3%	1.3%

The cell-free extract expressing β -ketoacyl-ACP reductase showed the NADPH-dependent ethyl 4-chloroacetoacetate-reduce activity and its specific activity was 0.133 U/mg protein. In contrast, the HB101 strain containing no plasmid pSE-BSR1 showed almost no ethyl 4-chloroacetoacetate-reducing activity. The acetoacetyl-CoA-reducing activity was as low as about 22% of ethyl 4-chloroacetoacetate-reducing activity, revealing the difference from the enzyme derived from *Escherichia coli*.

EXAMPLE 10

Oxidizing Activity of β -Ketoacyl-ACP Reductase
derived from *Bacillus subtilis*

The oxidizing activity of the cell-free extract obtained in Example 8 was assayed using ethyl (S)- or (R)-4-chloro-3-hydroxybutyrate as the substrate in the same manner as in Example 4. The results are shown in Table 2. The cell extract showed almost no activity for either substrate. In table 2, ECHB represents ethyl 4-chloro-3-hydroxybutyrate.

EXAMPLE 11

Stereoselectivity of β -ketoacyl-ACP Reductase derived from *Bacillus subtilis*

A reaction mixture (1 ml) containing 200 mM potassium phosphate buffer (pH 6.5), 1 mM NADP⁺, 2% ethyl 4-chloroacetoacetate (122 mM), β -ketoacyl-ACP reductase (0.8 U) prepared in Example 8, 250 mM glucose, and glucose dehydrogenase (3.2 U) (Wako Pure Chemical) was incubated at 25°C for one day. Analysis was performed by the method according to Example 5, indicating that ethyl 4-chloro-3-hydroxybutyrate was quantitatively synthesized with the optical purity of 98.1% ee (S) or more.

EXAMPLE 12

Isolation of Acetoacetyl-CoA Reductase Gene from *Ralstonia eutropha*

Ralstonia eutropha DSM 531 was cultured in a bouillon medium (containing beef extracts 5.0 g, peptone 15.0 g, NaCl 5.0 g, and K₂HPO₄ 5.0 g/l) and the chromosomal DNA was prepared from the microbial cells thus obtained using a Qiagen Genomic-tip (Qiagen).

For the PCR cloning of the acetoacetyl-CoA reductase gene (phbB) of *Ralstonia eutropha*, the primers AER-ATG1 (5'-AGTGGATCCAATGACTCAGCGCATTGCGTA-3', SEQ ID NO: 11) and AER-TAA1 (5'-AACAAAGCTTCTCGAGTTAGCCCATATGCAGGCCGC-3', SEQ ID NO: 12) were

synthesized based on the sequences at the 5'- and 3'-ends of the structural gene.

Using the chromosomal DNA of *Ralstonia eutropha* as the template and the AER-ATG1 and AER-TAA1 primers, 30 cycles of PCR (95°C for 30 sec, 50°C for 1 min and 75°C for 2 min) were performed to obtain the amplified DNA fragments.

The thus-obtained DNA fragments were digested with BamHI and HindIII. The plasmid vector pSE420 prepared by the method of Example 1 was digested with BamHI and HindIII and the digestion product was ligated to the above PCR-amplified fragments digested with the same two restriction enzymes using T4 DNA ligase to obtain the plasmid pSE-AER1.

The DNA insert in the plasmid thus obtained were sequenced and identified as the phbB gene.

EXAMPLE 13

Expression of Acetoacetyl-CoA Reductase Gene derived from *Ralstonia eutropha*

Escherichia coli HB101 strain was transformed with pSE-AER1 and the resulting transformant (*E. coli* HB101 (pSE-AER1)) was cultured in the LB medium (7 ml) containing ampicillin (50 mg/ml) overnight. IPTG was added to the medium to 0.1 mM and the culture was further incubated for 5 h.

Bacterial cells thus obtained were collected, disrupted with a Minibeatbeater 8 (BIOSPEC Inc.), and centrifuged to obtain the supernatant as the cell-free extract.

EXAMPLE 14

Reducing Activity of Acetoacetyl-CoA Reductase derived from *Ralstonia eutropha*

The reducing activity of the cell-free extract obtained in Example 13 was assayed using ethyl 4-chloroacetoacetate and acetoacetyl-CoA as the substrate by the method according to Example 3. The results are shown in Table 3.

Table 3

Substrate	AASCoA	ECAA		S-ECHB	R-ECHB
Coenzyme	NADPH	NADPH	NADH	NADP ⁺	NADP ⁺
U/mg	0.984	2.50	0.056	0	0
Relative activity	100%	255%	5.7%	0%	0%

The cell-free extract expressing acetoacetyl-CoA reductase showed the NADPH-dependent ethyl 4-chloroacetoacetate-reducing activity and its specific activity was 2.50 U/mg protein. In contrast, the HB101 strain containing no plasmid pSE-AER1 showed almost no ethyl 4-chloroacetoacetate-reducing activity.

The reducing activity with acetoacetyl-CoA was as low as about 39% of that with ethyl 4-chloroacetoacetate.

EXAMPLE 15

Oxidizing Activity of Acetoacetyl-CoA Reductase derived from *Ralstonia eutropha*

The oxidizing activity of the cell-free extract obtained in Example 13 was assayed using ethyl (S)- or (R)-4-chloro-3-hydroxybutyrate as the substrate in the same manner as in Example 4. The results are shown in Table 3. The cell extract showed almost no oxidizing activity for either substrate.

EXAMPLE 16

Stereoselectivity of Acetoacetyl-CoA Reductase from *Ralstonia eutropha*

A reaction mixture (1 ml) containing 100 mM potassium phosphate buffer (pH 6.5), 1 mM NADP⁺, 2% (122 mM) ethyl 4-chloroacetoacetate, acetoacetyl-CoA reductase (1 U) prepared in Example 13, 243 mM glucose, and glucose dehydrogenase (2.8 U) (Wako Pure Chemical) was incubated at 25°C overnight. Analysis was performed by the method according to Example 5, revealing a synthesis of ethyl 4-chloro-3-hydroxybutyrate with the optical purity of 99% ee (S) or more in a yield of 67%.

EXAMPLE 17

Construction of the Coexpression Plasmid pSE420D

The plasmid pSE420B constructed in Example 1 was digested with MunI and SpeI and ligated with the annealing product of the synthetic DNA SE420D-S (SEQ ID NO 13: AATTCTCGAGTAATCTAGAGGAATTCTAAAA) and the synthetic DNA SE420D-A (SEQ ID NO 14: CTAGTTTTAGAATTCCTCTAGATTACTCGAG) using T4 DNA ligase to obtain the plasmid pSE420D. The restriction map of pSE420D is shown in Figure 1.

EXAMPLE 18

Cloning of Glucose Dehydrogenase Gene derived from *Bacillus subtilis*

In order to regenerate the reduced nicotinamide adenine dinucleotide phosphate, the glucose dehydrogenase gene derived from *Bacillus subtilis* (J. Bacteriol. 166, 238-243 (1986)) was cloned.

For the PCR cloning of only the open reading frame portion of the glucose dehydrogenase gene based on the nucleotide sequence described in the literature, the primers BSG-ATG1 (SEQ ID NO 15: GAGGAATTCATACATGTATCCAGATTTAAAAGGAA) and BSG-TAA2 (SEQ ID NO 16: GGTAAGCTTTCATTAACCGCGGCTGCCTG) were synthesized based on the

sequences at the 5'- and 3'-ends of the structural gene.

Using the chromosomal DNA of *Bacillus subtilis* prepared in Example 7 as the template and the BSG-ATG1 and BSG-TAA2 primers, 30 cycles of PCR (95°C for 30 sec, 50°C for 1 min, and 75°C for 3 min and 15 sec) were performed, obtaining the amplified DNA fragments.

The thus-obtained DNA fragments were digested with EcoRI and HindIII and the digestion product was ligated with the plasmid vector pSE420L constructed in Example 17 digested with the same two restriction enzymes using T4 DNA ligase to obtain the plasmid pSE-BSG1.

Analysis of the nucleotide sequence of the DNA insert resulted in the perfect coincidence with that listed in the database (DDBJ Accession No. M12276).

The nucleotide sequence of the glucose dehydrogenase gene thus obtained is shown in SEQ ID NO: 17 and the amino acid sequence of the protein encoded by the gene in SEQ ID NO: 18.

EXAMPLE 19

Construction of the plasmid pSG-ECR1 coexpressing the β -ketoacyl-ACP Reductase Gene derived from *Escherichia coli* and the Glucose Dehydrogenase Gene derived from *Bacillus subtilis*

The plasmid pSE-ECR1 constructed in Example 1 was digested with BamHI and XhoI to obtain the DNA fragment containing the β -ketoacyl-ACP reductase gene derived from *Escherichia coli*.

The plasmid pSE-BSG1 containing the glucose dehydrogenase gene derived from *Bacillus subtilis* constructed in Example 17 was digested with BamHI and XhoI, ligated to the above DNA fragment containing the β -ketoacyl-ACP reductase gene derived from *Escherichia coli* using T4 DNA ligase to obtain the plasmid pSE-ECR1 capable of coexpressing

glucose dehydrogenase and β -ketoacyl-ACP reductase. The restriction map of the plasmid pSE-ECR1 is shown in Figure 2.

EXAMPLE 20

Construction of the plasmid pSG-ECR1 coexpressing the β -ketoacyl-ACP Reductase Gene derived from *Bacillus subtilis* and the Glucose Dehydrogenase Gene derived from *Bacillus subtilis*

The plasmid pSE-ECR1 constructed in Example 7 was digested with NcoI and XhoI to obtain the DNA fragment containing the β -ketoacyl-ACP reductase gene derived from *Bacillus subtilis*.

The plasmid pSE-BSG1 containing the glucose dehydrogenase gene derived from *Bacillus subtilis* constructed in Example 17 was digested with BamHI and XhoI, ligated to the above DNA fragment containing the β -ketoacyl-ACP reductase gene derived from *Bacillus subtilis* using T4 DNA ligase to obtain the plasmid pSG-BSR1 capable of coexpressing glucose dehydrogenase and β -ketoacyl-ACP reductase. The restriction map of the plasmid pSG-BSR1 is shown in Figure 3.

EXAMPLE 21

Construction of Plasmid pSG-AER1 coexpressing the Acetoacetyl-CoA Reductase Gene derived from *Ralstonia eutropha* and the Glucose Dehydrogenase Gene derived from *Bacillus subtilis*

The plasmid pSE-AER1 constructed in Example 12 was digested with BamHI and XhoI to obtain the DNA fragment containing the acetoacetyl-CoA reductase gene derived from *Ralstonia eutropha*.

The plasmid pSE-BSG1 containing the glucose dehydrogenase gene derived from *Bacillus subtilis* constructed in Example 17 was digested with NcoI and XhoI, ligated to the above DNA fragment containing the acetoacetyl-CoA reductase gene derived from *Ralstonia eutropha* using

T4 DNA ligase to obtain the plasmid pSG-AER1 capable of coexpressing glucose dehydrogenase and acetoacetyl-CoA reductase. The restriction map of the plasmid pSG-AER1 is shown in Figure 4.

EXAMPLE 22

Coexpression of Glucose Dehydrogenase derived from *Bacillus subtilis* and β -ketoacyl-ACP Reductase derived from *Escherichia coli* or β -ketoacyl-ACP Reductase derived from *Bacillus subtilis* or Acetoacetyl-CoA Reductase derived from *Ralstonia eutropha*

Escherichia coli HB101 strain was transformed with the plasmids pSG-ECR1, pSG-BSR1, or pSG-AER1 to coexpress glucose dehydrogenase derived from *Bacillus subtilis* with β -ketoacyl-ACP reductase derived from *Escherichia coli* or with β -ketoacyl-ACP reductase derived from *Bacillus subtilis* or with acetoacetyl-CoA reductase derived from *Ralstonia eutropha*.

Recombinant *Escherichia coli* transformed with each plasmid was inoculated to the 2xYT medium (containing Bacto-Tryptone 20 g, Bacto-Yeast extract 10 g, and NaCl 10 g/l), cultured at 30°C overnight. Adding 0.1 mM IPTG to the medium, the culturing was further performed for 4 h.

Three kinds of *Escherichia coli* transformants thus obtained were collected and subjected to the enzyme activity assay and the reducing reaction of ethyl 4-chloroacetoacetate.

EXAMPLE 23

Enzymatic Activity of *Escherichia coli* cells transformed with pSG-ECR1, pSG-BSR1 and pSG-AER1

Escherichia coli cells (corresponding to 2 ml of the culture) transformed with pSG-ECR1, pSG-BSR1, or pSG-AER1 prepared in Example

21 were separately suspended in 0.25 ml of a cell disrupting solution (containing 100 mM potassium phosphate buffer (pH 6.5), 0.02% 2-mercaptoethanol, and 0.5 M sodium chloride). The resulting solution was treated in a close ultrasonicator UCD-200TM (Cosmobio) for 3 min to disrupt the cells. The sonicated cell suspension was centrifuged and the supernatant was collected as the crude enzyme solution, which was subjected to the enzyme activity assay.

The ethyl 4-chloroacetoacetate-reducing activity was assayed in the same manner as in Example 3.

Glucose dehydrogenase activity was assayed in a reaction solution containing 100 mM potassium phosphate bufferr (pH 6.5), 2.5 mM NAD⁺, 100 mM D-glucose, and the enzyme at 25°C.

One unit of each enzyme was defined as the amount of the enzyme catalyzing the formation of 1 μ mol NADH per min under the above-described reaction conditions.

Each enzyme activity and SECHB productivity of the crude enzyme solution obtained from recombinant *E. coli* containing pSG-ECR1, pSG-BSR1, or pSG-AER1 are shown in Table 4.

Table 4

Plasmid	Enzyme activity (U/ml-br)		SECHB synthesis	
	GlcDH	ECAA-R	(g/l)	ee(%)S
pSG-ECR1	0.476	0.151	20.3	93.7
pSG-BSR1	4.089	0.203	42.3	98.0
pSG-AER1	3.717	1.475	42.8	99.3

U/ml-br: enzyme activity per ml of the culture

GlcDH: glucose dehydrogenase

ECAA-R: ethyl 4-chloroacetoacetate reductase

SECHB: ethyl (S)-4-chloro-3-hydroxybutyrate

E. coli cells with no plasmid have neither glucose dehydrogenase activity nor ethyl 4-chloroacetoacetate reductase activity, while recombinant *E. coli* cells with pSG-ECR1, pSG-BSR1, or pSG-AER1 showed both enzymatic activities, indicating expression of the two enzyme genes introduced in the plasmids.

EXAMPLE 24

Synthesis of Ethyl (S)-4-chloro-3-hydroxybutyrate using *Escherichia coli* transformed with pSG-ECR1, pSG-BSR1, or pSG-AER1

Ethyl 4-chloroacetoacetate was reduced using *Escherichia coli* containing pSG-ECR1, pSG-BSR1, or pSG-AER1 prepared in Example 21.

A reaction solution (20 ml) containing each *Escherichia coli* cell prepared from 20 ml of the culture, 200 mM potassium phosphate buffer (pH 6.5), 4% ethyl 4-chloroacetoacetate, 486 mM D-glucose, and 1 mM NADP⁺ was incubated at 25°C under stirring overnight.

The amount and the optical purity of ethyl (S)-4-chloro-3-hydroxybutyrate were determined in the same manner as in Example 5. The results are shown in Table 4. As shown in Table 4, all of the three kinds of the *E. coli* transformants could produce (S)-4-chloro-3-hydroxybutyrate efficiently with high stereoselectivity.

SEQUENCE LISTING

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<120> Method for producing optically active
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<130> D1-003DP2

<150> JP 1998-126507

<151> 1998-05-08

<150> JP 1998-300178

<151> 1998-10-21

<160> 12

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<210> 1

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<213> Escherichia coli

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35 40 45

Leu Gly Ala Asn Gly Lys Gly Leu Met Leu Asn Val Thr Asp Pro Ala
50 55 60

Ser Ile Glu Ser Val Leu Glu Lys Ile Arg Ala Glu Phe Gly Glu Val
65 70 75 80

Asp Ile Leu Val Asn Asn Ala Gly Ile Thr Arg Asp Asn Leu Leu Met
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Arg Met Lys Asp Glu Glu Trp Asn Asp Ile Ile Glu Thr Asn Leu Ser
100 105 110

Ser Val Phe Arg Leu Ser Lys Ala Val Met Arg Ala Met Met Lys Lys
115 120 125

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			100					105					110			
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Asp Arg Gly Trp Gly Arg Ile Val Asn Ile Ser Ser Val Asn Gly Gln
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Ala Ile Arg Gln Asp Val Leu Asp Lys Ile Val Ala Thr Ile Pro Val
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Lys Arg Leu Gly Leu Pro Glu Glu Ile Ala Ser Ile Cys Ala Trp Leu
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Phe Arg Lys Met Thr Arg Ala Asp Trp Asp Ala Val Ile Asp Thr Asn	
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Asp Lys Val Ile Gly Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg	
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gaa gcg att aaa tat ttc gta gaa aac gat atc aag gga aat gtc att	434
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What is claimed is:

1. A method for producing (S)-4-halo-3-hydroxybutyric acid ester comprising asymmetrically reducing 4-halo-acetoacetic acid ester or its derivatives with β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase.

2. The method of claim 1, wherein said β -ketoacyl carrier protein reductase is derived from *Escherihia coli*.

3. The method of claim 1, wherein said β -ketoacyl carrier protein reductase is selected from the group consisting of:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 1;
- (b) a protein comprising a modified amino acid sequence of SEQ ID NO: 1 in which one or more amino acid residues are added, deleted, or substituted and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester; and
- (c) a protein encoded by DNA hybridizable with the DNA comprising the nucleotide sequence of SEQ ID NO: 2 and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester.

4. The method of claim 1, wherein said β -ketoacyl-acyl carrier protein reductase is derived from microorganisms belonging to the genus *Bacillus*.

5. The method of claim 4, wherein said β -ketoacyl-acyl carrier protein reductase is derived from *Bacillus subtilis*.

6. The method of claim 1, wherein said β -ketoacyl-acyl carrier protein reductase is selected from the group consisting of:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 5;
- (b) a protein comprising a modified amino acid sequence of SEQ ID NO: 5 in which one or more amino acid residues are added, deleted,

or substituted and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester; and

(c) a protein encoded by DNA hybridizable with the DNA comprising the nucleotide sequence of SEQ ID NO: 6 and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester.

7. A method for producing (S)-4-halo-3-hydroxybutyric acid ester comprising asymmetrically reducing 4-halo-acetoacetic acid ester or its derivatives with acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid biosynthesis system.

8. A method of claim 7, wherein said acetoacetyl-CoA reductase is derived from microorganisms belonging to the genus *Ralstonia*.

9. The method of claim 8, wherein said acetoacetyl-CoA reductase is derived from *Ralstonia eutropha*.

10. The method of claim 7, wherein said acetoacetyl-CoA reductase is selected from the group consisting of:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 9;

(b) a protein comprising a modified amino acid sequence of SEQ ID NO: 9 in which one or more amino acid residues are added, deleted, or substituted and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester; and

(c) a protein encoded by DNA hybridizable with the DNA comprising the nucleotide sequence of SEQ ID NO: 10 and capable of asymmetrically reducing 4-haloacetoacetic acid ester or its derivatives to produce (S)-4-halo-3-hydroxybutyric acid ester.

11. The method of claim 1, wherein said 4-haloacetoacetic

acid ester is 4-chloroacetoacetic acid ester.

12. The method of claim 7, wherein said 4-haloacetoacetic acid ester is 4-chloroacetoacetic acid ester.

13. The method of claim 1, wherein said 4-haloacetoacetic acid ester is ethyl 4-chloroacetoacetate.

14. The method of claim 7, wherein said 4-haloacetoacetic acid ester is ethyl 4-chloroacetoacetate.

15. The method of claim 1, wherein said method uses a microorganism capable of producing β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase and an enzyme catalyzing production of NAD(P)H from NAD(P)⁺.

16. The method of claim 15, wherein said microorganism is a recombinant microorganism into which heterologous or homologous DNA encoding the β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase and heterologous or homologous DNA encoding an enzyme catalyzing production of NAD(P)H from NAD(P)⁺, and is capable of expressing both enzymes.

17. The method of claim 16, wherein said microorganism is *Escherihia coli*.

18. The method of any one of claims 15, wherein said enzyme catalyzing production of NAD(P)H from NAD(P)⁺ is glucose dehydrogenase.

19. The method of claim 7, wherein said method uses a microorganism capable of producing acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid biosynthesis system and an enzyme catalyzing production of NAD(P)H from NAD(P)⁺.

20. The method of claim 19, wherein said microorganism is a recombinant microorganism into which heterologous or homologous DNA encoding acetoacetyl-CoA reductase constituting the poly- β -

hydroxy fatty acid biosynthesis system and heterologous or homologous DNA encoding an enzyme catalyzing production of NAD(P)H from NAD(P)⁺ and capable of expressing both enzymes.

21. The method of claim 20, wherein said microorganism is *Escherichia coli*.

22. The method of any one of claims 19, wherein said enzyme catalyzing production of NAD(P)H from NAD(P)⁺ is glucose dehydrogenase.

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ABSTRACT OF THE DISCLOSURE

The present invention provides a method for preparing (S)-4-halo-3-hydroxybutyric acid ester comprises asymmetric reduction of 4-halo-acetoacetic acid ester using β -ketoacyl-acylcarrier protein reductase constituting Type II fatty acid synthase or acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid synthesis system. β -ketoacyl-acyl carrier protein reductase constituting Type II fatty acid synthase or acetoacetyl-CoA reductase constituting the poly- β -hydroxy fatty acid synthesis system has a extremely high reducing activity as well as stereoselectivity for (S)-4-chloro-3-hydroxybutyric acid ester. In addition, the enzyme exhibits almost no oxidizing activity toward either configuration of ethyl 4-chloro-3-hydroxybutyrate, performing only the reducing reaction of ethyl 4-chloroacetoacetate. Therefore, this enzyme can be used to efficiently produce (S)-4-halo-3-hydroxybutyric acid ester.

Fig. 1

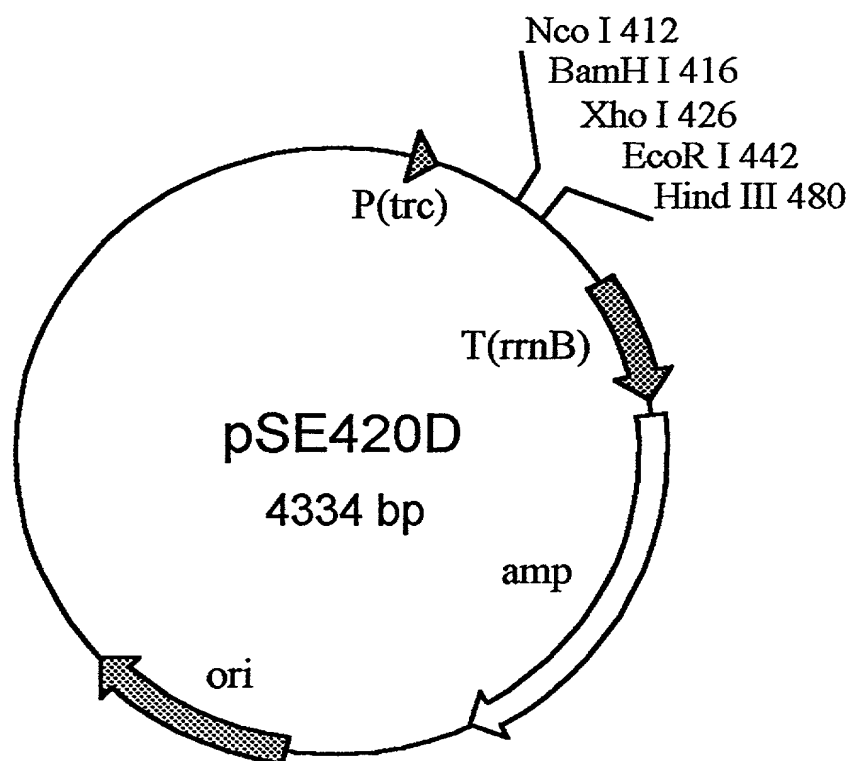


Fig. 2

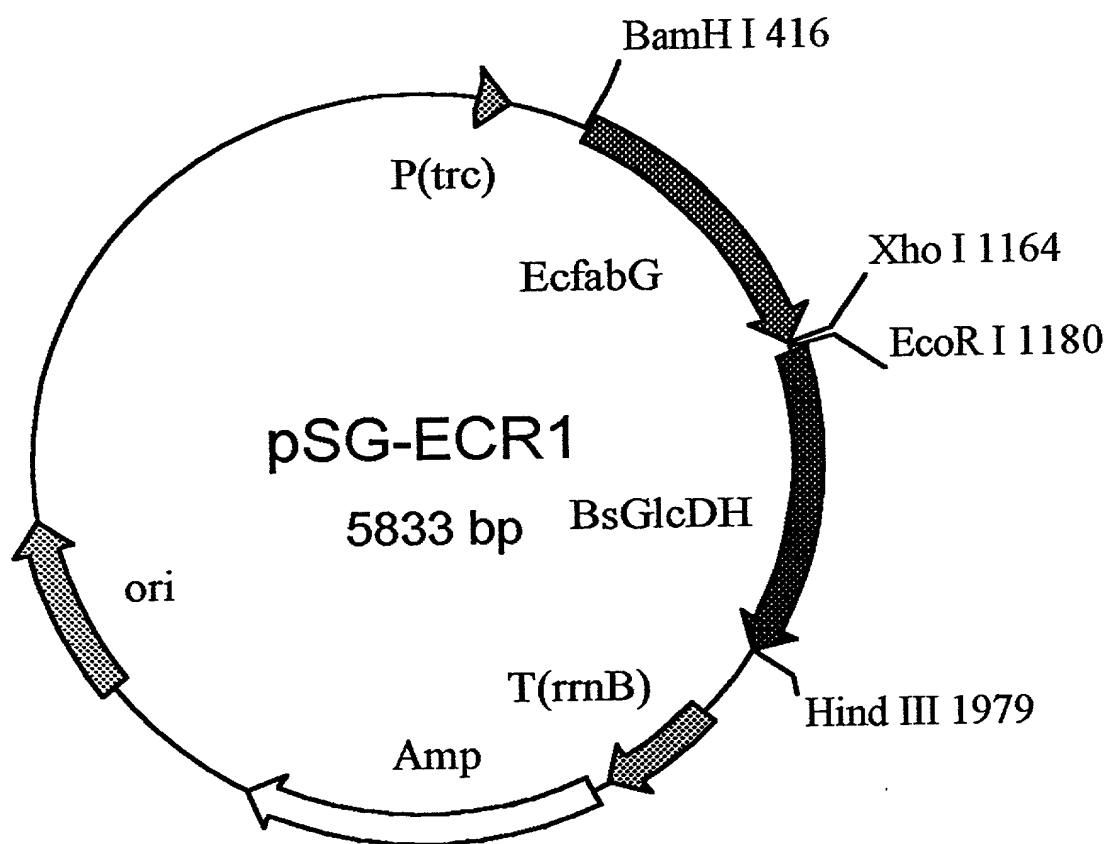


Fig. 3

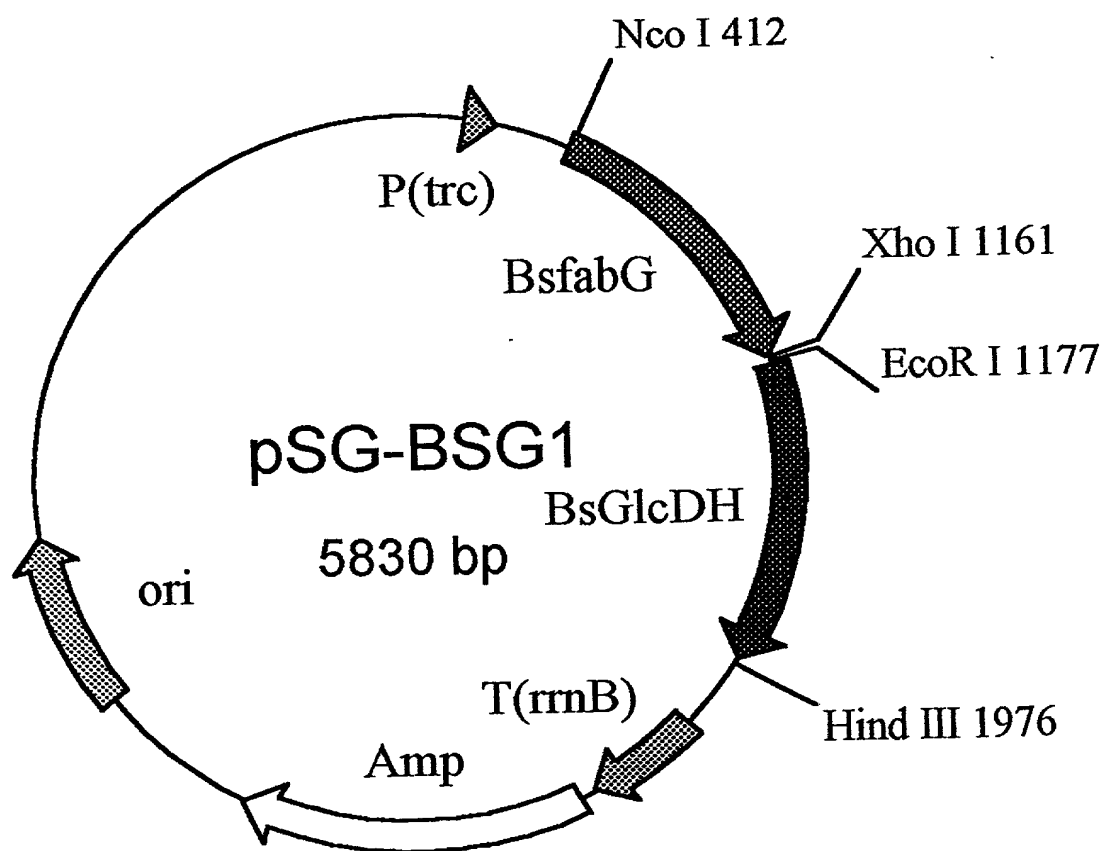
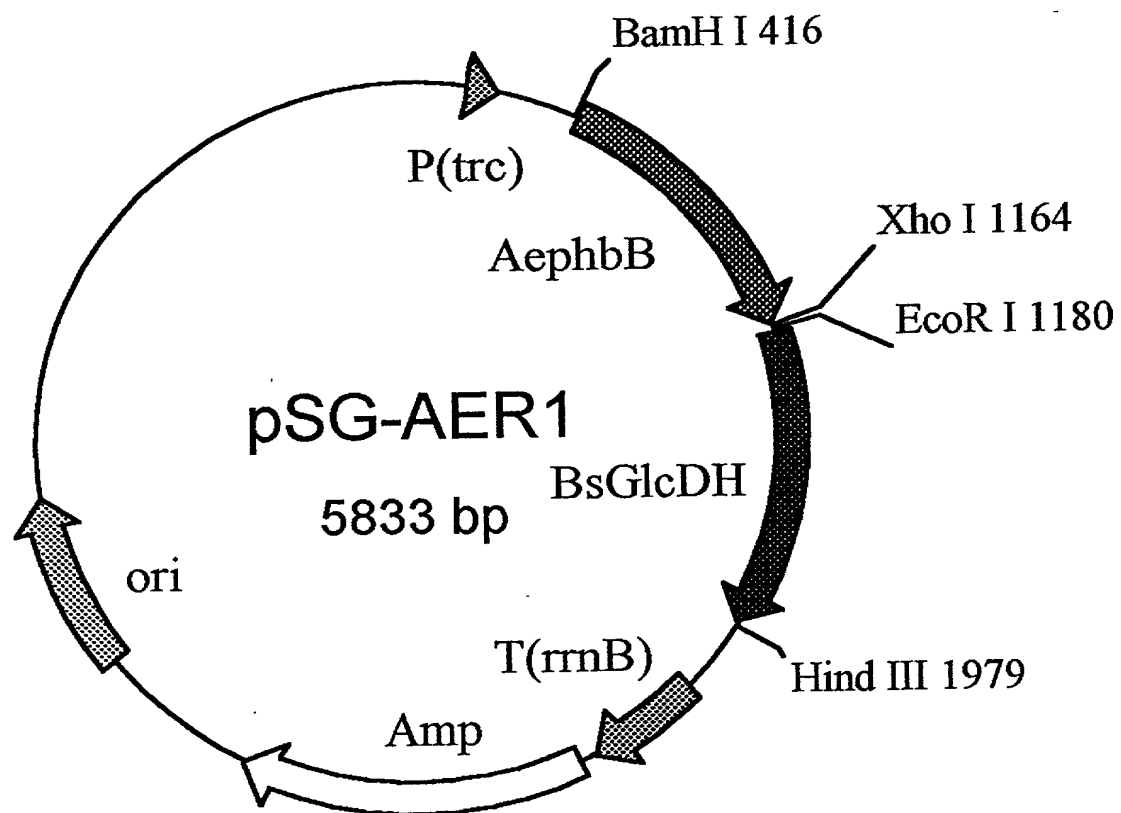


Fig. 4



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR PRODUCING OPTICALLY ACTIVE 4-HALO-3-HYDROXYBUTYRIC ACID ESTER, the specification of which

- ☒ is attached hereto.
☐ was filed on _____ as Application Serial No. _____
and was amended on _____.
☐ was described and claimed in PCT International Application No. _____
filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

COUNTRY	APPLICATION NO.	FILING DATE	PRIORITY CLAIMED
Japan	10-126507	May 8, 1998	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Japan	10-300178	October 21, 1998	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Japan	11-98205	April 5, 1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Janis K. Fraser, Reg. No. 34,819; J. Peter Fasse, Reg. No. 32,983; John W. Freeman, Reg. No. 29,066; Timothy A. French, Reg. No. 30,175; Eldora L. Ellison, Reg. No. 39,967; Anita L. Meiklejohn, Reg. No. 35,283; and Ralph A. Mittelberger, Reg. No. 33,195.

Address all telephone calls to Janis K. Fraser at telephone number 617/542-5070.

Address all correspondence to Janis K. Fraser, Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

Full Name of Inventor: Hiroaki Yamamoto

Inventor's Signature: _____ Date: _____

Residence Address: Ibaraki, Japan

Citizen of: Japan

Post Office Address: 1-14-14-103, Sengen, Tsukuba-Shi, Ibaraki 305-0047 Japan

371273.B11

SEQUENCE LISTING

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Ala	Gly	Val	Ile	Gly	Leu	Thr	Lys	Ser	Ser	Ala	Lys	Glu	Leu	Ala	Ser	
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cga aat att acg gta aac gca ata gcg cca gga ttt atc tca act gat	576
Arg Asn Ile Thr Val Asn Ala Ile Ala Pro Gly Phe Ile Ser Thr Asp	
180 185 190	
atg aca gat aaa ctt gca aaa gac gtt caa gac gaa atg ctg aaa caa	624
Met Thr Asp Lys Leu Ala Lys Asp Val Gln Asp Glu Met Leu Lys Gln	
195 200 205	
att ccg ctc gcg cgc ttt ggt gaa cct agc gat gtc agc agt gtt gtc	672
Ile Pro Leu Ala Arg Phe Gly Glu Pro Ser Asp Val Ser Ser Val Val	
210 215 220	
acg ttc cta got tca gag gga gct cgt tat atg aca ggc caa acg ctt	720
Thr Phe Leu Ala Ser Glu Gly Ala Arg Tyr Met Thr Gly Gln Thr Leu	
225 230 235 240	
cat att gac ggc gga atg gtg atg taa	747
His Ile Asp Gly Gly Met Val Met	
245	

<210> 7
 <211> 33
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Artificially synthesized primer sequence

<400> 7	33
ggaccatgga tatgcttaat gataaaacgg cta	

<210> 8
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Artificially synthesized primer sequence

<400> 8	34
gagaagcttc tcgagttaca tcaccattcc gccg	

<210> 9
 <211> 246
 <212> PRT
 <213> Ralstonia eutropha

<400> 9	
Met Thr Gln Arg Ile Ala Tyr Val Thr Gly Gly Met Gly Gly Ile Gly	
1 5 10 15	
Thr Ala Ile Cys Gln Arg Leu Ala Lys Asp Gly Phe Arg Val Val Ala	
20 25 30	
Gly Cys Gly Pro Asn Ser Pro Arg Glu Lys Trp Leu Glu Gln Gln	
35 40 45	
Lys Ala Leu Gly Phe Asp Phe Ile Ala Ser Glu Gly Asn Val Ala Asp	
50 55 60	
Trp Asp Ser Thr Lys Thr Ala Phe Asp Lys Val Lys Ser Glu Val Gly	
65 70 75 80	

Glu Val Asp Val Leu Ile Asn Asn Ala Gly Ile Thr Arg Asp Val Val
 85 90 95
 Phe Arg Lys Met Thr Arg Ala Asp Trp Asp Ala Val Ile Asp Thr Asn
 100 105 110
 Leu Thr Ser Leu Phe Asn Val Thr Lys Gln Val Ile Asp Gly Met Ala
 115 120 125
 Asp Arg Gly Trp Gly Arg Ile Val Asn Ile Ser Ser Val Asn Gly Gln
 130 135 140
 Lys Gly Gln Phe Gly Gln Thr Asn Tyr Ser Thr Ala Lys Ala Gly Leu
 145 150 155 160
 His Gly Phe Thr Met Ala Leu Ala Gln Glu Val Ala Thr Lys Gly Val
 165 170 175
 Thr Val Asn Thr Val Ser Pro Gly Tyr Ile Ala Thr Asp Met Val Lys
 180 185 190
 Ala Ile Arg Gln Asp Val Leu Asp Lys Ile Val Ala Thr Ile Pro Val
 195 200 205
 Lys Arg Leu Gly Leu Pro Glu Glu Ile Ala Ser Ile Cys Ala Trp Leu
 210 215 220
 Ser Ser Glu Glu Ser Gly Phe Ser Thr Gly Ala Asp Phe Ser Leu Asn
 225 230 235 240
 Gly Gly Leu His Met Gly
 245

<210> 10
 <211> 741
 <212> DNA
 <213> Ralstonia eutropha

<220>
 <221> CDS
 <222> (1)...(738)

<400> 10
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 Met Thr Gln Arg Ile Ala Tyr Val Thr Gly Gly Met Gly Gly Ile Gly
 1 5 10 15

 acc gcc att tgc cag cgg ctg gcc aag gat ggc ttt cgt gtg gtg gcc 96
 Thr Ala Ile Cys Gln Arg Leu Ala Lys Asp Gly Phe Arg Val Val Ala
 20 25 30

 ggt tgc ggc ccc aac tcg ccg cgc cgc gaa aag tgg ctg gag cag cag 144
 Gly Cys Gly Pro Asn Ser Pro Arg Arg Glu Lys Trp Leu Glu Gln Gln
 35 40 45

 aag gcc ctg ggc ttc gat ttc att gcc tcg gaa ggc aat gtg gct gac 192
 Lys Ala Leu Gly Phe Asp Phe Ile Ala Ser Glu Gly Asn Val Ala Asp
 50 55 60

 tgg gac tcg acc aag acc gca ttc gac aag gtc aag tcc gag gtc ggc 240
 Trp Asp Ser Thr Lys Thr Ala Phe Asp Lys Val Lys Ser Glu Val Gly
 65 70 75 80

 gag gtt gat gtg ctg atc aac aac gcc ggt atc acc cgc gac gtg gtg 288
 Glu Val Asp Val Leu Ile Asn Asn Ala Gly Ile Thr Arg Asp Val Val
 85 90 95

 ttc cgc aag atg acc cgc gcc gac tgg gat gcg gtg atc gac acc aac 336
 Phe Arg Lys Met Thr Arg Ala Asp Trp Asp Ala Val Ile Asp Thr Asn
 100 105 110

ctg acc tcg ctg ttc aac gtc acc aag cag gtg atc gac ggc atg gcc	384
Leu Thr Ser Leu Phe Asn Val Thr Lys Gln Val Ile Asp Gly Met Ala	
115 120 125	
gac cgt ggc tgg ggc cgc atc gtc aac atc tcg tcg gtg aac ggg cag	432
Asp Arg Gly Trp Gly Arg Ile Val Asn Ile Ser Ser Val Asn Gly Gln	
130 135 140	
aag ggc cag ttc ggc cag acc aac tac tcc acc gcc aag gcc ggc ctg	480
Lys Gly Gln Phe Gly Gln Thr Asn Tyr Ser Thr Ala Lys Ala Gly Leu	
145 150 155 160	
cat ggc ttc acc atg gca ctg gcg cag gaa gtg gcg acc aag ggc gtg	528
His Gly Phe Thr Met Ala Leu Ala Gln Glu Val Ala Thr Lys Gly Val	
165 170 175	
acc gtc aac acg gtc tct ccg ggc tat atc gcc acc gac atg gtc aag	576
Thr Val Asn Thr Val Ser Pro Gly Tyr Ile Ala Thr Asp Met Val Lys	
180 185 190	
gcg atc cgc cag gac gtg ctc gac aag atc gtc gcg acg atc ccg gtc	624
Ala Ile Arg Gln Asp Val Leu Asp Lys Ile Val Ala Thr Ile Pro Val	
195 200 205	
aag cgc ctg ggc ctg ccg gaa gag atc gcc tcg atc tgc gcc tgg ttg	672
Lys Arg Leu Gly Leu Pro Glu Glu Ile Ala Ser Ile Cys Ala Trp Leu	
210 215 220	
tcg tcg gag gag tcc ggt ttc tcg acc ggc gcc gac ttc tcg ctc aac	720
Ser Ser Glu Glu Ser Gly Phe Ser Thr Gly Ala Asp Phe Ser Leu Asn	
225 230 235 240	
ggc ggc ctg cat atg ggc taa	741
Gly Gly Leu His Met Gly	
245	

<210> 11
 <211> 30
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Artificially synthesized primer sequence

<400> 11
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<210> 12
 <211> 35
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Artificially synthesized primer sequence

<400> 12
 aacaagcttc tcgagttagc ccatatgcag gccgc 35

<210> 13
 <211> 31
 <212> DNA

<213> Artificial Sequence
 <220>
 <223> Artificially synthesized sequence
 <400> 13
 aattctcgag taatctagag gaattctaaa a 31
 <210> 14
 <211> 31
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Artificially synthesized sequence
 <400> 14
 ctagttag aattcctcta gattactcga g 31
 <210> 15
 <211> 35
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Artificially synthesized primer sequence
 <400> 15
 gaggaattca tacatgtatc cagatttaaa aggaa 35
 <210> 16
 <211> 30
 <212> DNA
 <213> Artificial Sequence
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 <223> Artificially synthesized primer sequence
 <400> 16
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 <210> 17
 <211> 806
 <212> DNA
 <213> Bacillus subtilis
 <220>
 <221> CDS
 <222> (12)...(794)
 <400> 17
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 Met Tyr Pro Asp Leu Lys Gly Lys Val Val Ala Ile Thr
 1 5 10
 gga gct gct tca ggg ctc gga aag gcg atg gcc att cgc ttc ggc aag 98
 Gly Ala Ala Ser Gly Leu Gly Lys Ala Met Ala Ile Arg Phe Gly Lys
 15 20 25
 gag cag gca aaa gtg gtt atc aac tat tat agt aat aaa caa gat ccg 146
 Glu Gln Ala Lys Val Val Ile Asn Tyr Tyr Ser Asn Lys Gln Asp Pro
 30 35 40 45

aac gag gta aaa gaa gag gtc atc aag gcg ggc ggt gaa gct gtt gtc Asn Glu Val Lys Glu Val Ile Lys Ala Gly Gly Glu Ala Val Val 50 55 60	194
gtc caa gga gat gtc acg aaa gag gaa gat gta aaa aat atc gtg caa Val Gln Gly Asp Val Thr Lys Glu Glu Asp Val Lys Asn Ile Val Gln 65 70 75	242
acg gca att aag gag ttc ggc aca ctc gat att atg att aat aat gcc Thr Ala Ile Lys Glu Phe Gly Thr Leu Asp Ile Met Ile Asn Asn Ala 80 85 90	290
ggt ctt gaa aat cct gtg cca tct cac gaa atg ccg ctc aag gat tgg Gly Leu Glu Asn Pro Val Ser His Glu Met Pro Leu Lys Asp Trp 95 100 105	338
gat aaa gtc atc ggc acg aac tta acg ggt gcc ttt tta gga agc cgt Asp Lys Val Ile Gly Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg 110 115 120 125	386
gaa gcg att aaa tat ttc gta gaa aac gat atc aag gga aat gtc att Glu Ala Ile Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly Asn Val Ile 130 135 140	434
aac atg tcc agt gtg cac gaa gtg att cct tgg ccg tta ttt gtc cac Asn Met Ser Ser Val His Glu Val Ile Pro Trp Pro Leu Phe Val His 145 150 155	482
tat gcg gca agt aaa ggc ggg ata aag ctg atg aca gaa aca tta gcg Tyr Ala Ala Ser Lys Gly Gly Ile Lys Leu Met Thr Glu Thr Leu Ala 160 165 170	530
ttg gaa tac gcg ccg aag ggc att cgc gtc aat aat att ggg cca ggt Leu Glu Tyr Ala Pro Lys Gly Ile Arg Val Asn Asn Ile Gly Pro Gly 175 180 185	578
gcg atc aac acg cca atc aat gct gaa aaa ttc gct gac cct aaa cag Ala Ile Asn Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp Pro Lys Gln 190 195 200 205	626
aaa gct gat gta gaa agc atg att cca atg gga tat atc ggc gaa ccg Lys Ala Asp Val Glu Ser Met Ile Pro Met Gly Tyr Ile Gly Glu Pro 210 215 220	674
gag gag atc gcc gca gta gca gcc tgg ctt gct tcg aag gaa gcc agc Glu Glu Ile Ala Ala Val Ala Ala Trp Leu Ala Ser Lys Glu Ala Ser 225 230 235	722
tac gtc aca ggc atc acg tta ttc gcg gac ggc ggt atg aca caa tat Tyr Val Thr Gly Ile Thr Leu Phe Ala Asp Gly Gly Met Thr Gln Tyr 240 245 250	770
cct tca ttc cag gca ggc cgc ggt taatgaaagc tt Pro Ser Phe Gln Ala Gly Arg Gly 255 260	806

<210> 18
 <211> 261
 <212> PRT
 <213> Bacillus subtilis

